PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF *VIOLA ODORATA (LINN) F*OR ITS POTENTIAL IN EXPERIMENTAL INDUCED NEUROPATHIC PAIN IN RATS

SYNOPSIS OF THE THESIS

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DECLARATION

I hereby declare that the dissertation synopsis entitled "**Phytochemical and Pharmacological Screening of *Viola Odorata (Linn)* for Its Potential in Experimental Induced Neuropathic Pain in Rats**" to be submitted for the partial fulfillment of the Degree of Doctor of Philosophy is my original work and the dissertation has not formed the basis for the award of any degree, diploma, associateship or fellowship of similar other titles. It has not been submitted to any other University or Institution for the award of any degree or diploma.

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CHAPTER 1: INTRODUCTION

Neuropathic pain (NP) is a state of manifestation of multiple and varied disorders that affects both peripheral and central sensitization mechanisms.[1] It disturbs somatosensory components of the nervous system and leads to various physicochemical changes.[2] These physiological and biochemical changes in the nervous system result in morphological & functional adaptation to external stimuli and play a vital role in the generation and maintenance of pain symptoms.[2] [3] Injury to the peripheral nervous system generates secondary morphological and chemical changes in dorsal regulatory ganglions (DRG) which includes the formation of new ion channels, chemicals, and receptors at the cellular level, which ultimately results in hyperexcitability of neurons and leads to neuropathic pain. [4]

Beside it, many pathological conditions including cancer, AIDS, long-standing diabetes, lumbar disc syndrome, Autoimmune disorders, Viral infections, herpes infection, multiple sclerosis, and stroke can damage nerves which further generates neuropathic pain. More specifically neuropathic pain can be defined as pain caused by a lesion or disease of the somatosensory system including peripheral fibers (Aβ, Aδ, and C fibers) and characterized by allodynia and hyperalgesia. [2] Further neuropathic pain is also linked with augmented drug prescriptions and visits to health care providers. Increased prescription and visits to health care may lead to a set of symptoms including burning and electrical-like sensations, and pain resulting from non-painful stimulations (such as light touching); these symptoms may persist and tend to become chronic and respond less to pain medications. Patients with neuropathic pain may suffer sleep disturbances, anxiety, and depression. Neuropathic pain is one of the major disturbing conditions with long-term impairment of the affected patient's social life and overall quality of life. Quality of life is more impaired in patients with chronic neuropathic pain when compared with patients with chronic non-neuropathic pain that does not come from damaged or irritated nerves.[5]

Over 7% to 9% of the world population is affected by this kind of pain. Several Indian epidemiological studies had also shown a wide occurrence of neuropathic pain, varying from 5 to 2400 per 10,000 population in different communities. Chronic neuropathic pain is more common in women (8% versus 5.7% in men) and patients >50 years of age (8.9% versus 5.6% in those <49 years of age), and mostly affects the lower back, lower limbs, neck, and upper limbs. Lumbar and cervical painful radiculopathies are probably the most frequent

cause of chronic neuropathic pain.[5, 6] Neuro-injury leads to alterations in ion channels (sodium, calcium, and potassium) within the affected nerves, which can include all types of afferent fibers which ultimately affect the spinal and brain sensory signaling. For example, increased expression and function of sodium channels at the spinal cord terminus of the sensory nerves (mirrored by an enhanced expression of the α2δ subunit of calcium channels) lead to increased excitability, signal transduction, and release of excitatory neurotransmitters.[7] If an afferent fiber is disconnected from the periphery due to an injury or a lesion, there will be a sensory loss. However, the remnants of the fibers at the injury site can generate inflammatory and ectopic activity and hence can result in painful sensations in the numb area. The changed inputs into the spinal cord coupled with increased calcium channel function (through higher expression in the nerve terminal) result in increased neurotransmitter release and elevated excitatory synaptic transmission in the nociceptive circuit.[8] Increased excitability of neurons enables low-threshold mechanosensitive Aβ and Aδ afferent fibers to activate second-order nociceptive neurons (which convey sensory information from the spinal cord to the brain) and enlarges their receptive fields so a given stimulus excites more second-order nociceptive neurons which further generates central sensitization. Specifically ongoing discharge of peripheral afferent fibers with concomitant release of excitatory amino acids and neuropeptides leads to postsynaptic changes in second-order nociceptive neurons, such as an excess of signaling due to phosphorylation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors.[9]

GABAergic (γ-aminobutyric acid) inhibitory interneurons can also cause a condition of hyperexcitability in between first and second-order neurons of pain signaling. Interneuron dysfunction contributes to an imbalance between descending inhibitions and excitations; specifically, neuropathy leads to a shift in excitation that now dominates.[10] Further inhibition of microglia and astrocytes, was also reported to alleviate the hypersensitivity and neuropathic pain[11]. Various studies had also reported the involvement of various inflammatory mediators including NFkB, TNF-α, IL-1β, and IL-**6** in peripheral and central sensitization in the experimental neuropathic pain.[12, 13] Besides it anti-nociceptive pathway originates in the brain stem and travels down to the spinal cord where they synapse with short interneurons in the dorsal horn by releasing serotonin and norepinephrine. The interneurons modulate the synapse between the first-order neuron and the second-order neuron by

releasing gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter.[14] Hence, pain cessation is the result of inhibition of synapses between first and second-order neurons, while pain enhancement may be the result of suppression of inhibitory synaptic connections. Numerous drugs such as tricyclic antidepressants, anticonvulsants, opioid drugs, and other drugs including capsaicin and lidocaine are presently existing in the market for attenuating neuropathic pain.[15] However, these drugs were reported to exhibit specific adverse effects including neuropathy.[16] Hence currently drug discovery for attenuating neuropathic pain are more focused on herbal formulation.

Several herbal plants had shown potential in alleviating pain without any significant adverse effects in both preclinical and clinical studies of neuropathic pain. [17] Plants that are under evaluation for their potential in alleviating neuropathic pain includes Acorus calamus, Artemisia dracunculus, Butea monosperma, Citrullus colocynthis, Curcuma longa, Crocus sativus, Elaeagnus Angustifolia, Ginkgo biloba, Mitragyna speciosa, Momordica charantia, Nigella sativa, Ocimum sanctum, Phyllanthus amarus, Pterodon pubescent Benth, Rubia cordifolia cannabis Sativa, and Salvia officinalis.[18] Moreover, the most pathways which are known to be involved in attenuating pain by herbal remedies are anti-oxidant activity, anti-inflammatory, anti-apoptotic, neuroprotective, GABA potentiation, serotonin pathway activation, and calcium ion channel inhibitory actions. Hence, the search for a new medicine of plant origin is the current requirement for treating neuropathic.

Plant selected for current work i.e., *Viola odorata L.* commonly known as sweet or common violet is a perennial herb, spreads with stolons. Leaves are orbicular-reniform to broadly ovate. Flowers have dark violet or white color. This herb belongs to the family Violaceae. Commonly plant is termed as Bānāfsāj or Banafshe in Persian or traditional Persian medicine (TPM). Herb is native to Asia, North Africa, and Europe, the plant has been widely distributed in the northern areas of the Himalayas including Himachal Pradesh [19] Traditionally plant is used for the treatment of cough, fever, common cold, headache, insomnia, epilepsy, constipation, palpitation, dyspnea, dysuria, and skin diseases.[20] Besides it, this herb plant has exerted various activities including anti-inflammatory, analgesic, antioxidant, diuretic, antihypertensive, and antibacterial as reported in current pharmacotherapy. Pharmacological activities of the herb are reported due to the presence of various phytoconstituents including saponins, salicylates, alkaloids, flavonoids, saponins,

tannins, phenolics, coumarins, phenolic glycosides, gaultheria, violutoside, saponins, flavonoids, and odoratine Flavonoids, saponins, alkaloids, and melatonin.[21, 22] Hence, the selected herb, Viola odorata L. was used principally in traditional Persian medicine and Ayurveda for giving attractive results in curing and rejuvenating healthy life against diseases, however lack of scientific data on neuroprotective and antinociceptive activity of *Viola odorata L.* against chronic constriction nerve injury (CCI) induced neuropathic pain or neuropathy gain my attention towards this research.

CHAPTER 2: LITERATURE REVIEW

2.1 ANATOMY OF PERIPHERAL NERVOUS SYSTEM (PNS)

The peripheral nervous system refers to the Neves or neurons functioning outside the brain and the spinal cord. A single nerve is made up of a cluster of axons. Numerous axons are insulated with a substance called myelin which is formed by concentric layers of lipid-rich myelinating cells including oligodendrocytes in the central nervous system & Schwann cells in the peripheral nervous system. The cross-section of the spinal cord indicates gray matter is spliced into Dorsal, ventral and lateral horns. Dorsal regulatory ganglion (DRG) (Sensory fiber system) and ventral regulatory ganglions (VRG) (Motor fiber system) when comes together in a boney intervertebral foramen of the spinal cord forms a spinal nerve and termed as PNS.[23] (Figure 1) Ventral and dorsal ramus is formed just outside the intervertebral foramen of the spinal cord after it is supplied to the limbs. Ventral and dorsal rami are branches of the mixed nerve; they both carry sensory and motor fibers. Dysfunction of motor neurons results in muscle weakness or paralysis. Dysfunction of sensory neurons results in abnormal or loss of sensation. A peripheral nerve trunk is comprised of axons and multiple neuron bundles surrounded by perineurium. On the development of the pathological conditions, nociceptors receive an unpleasant or nociceptive stimulus and transmit it to CNS in the form of electrical stimuli. The conduction of electrical impulses, in turn, depends upon the size, diameter, and presence or absence of the myelin sheath encased on primary afferent axons.[24]

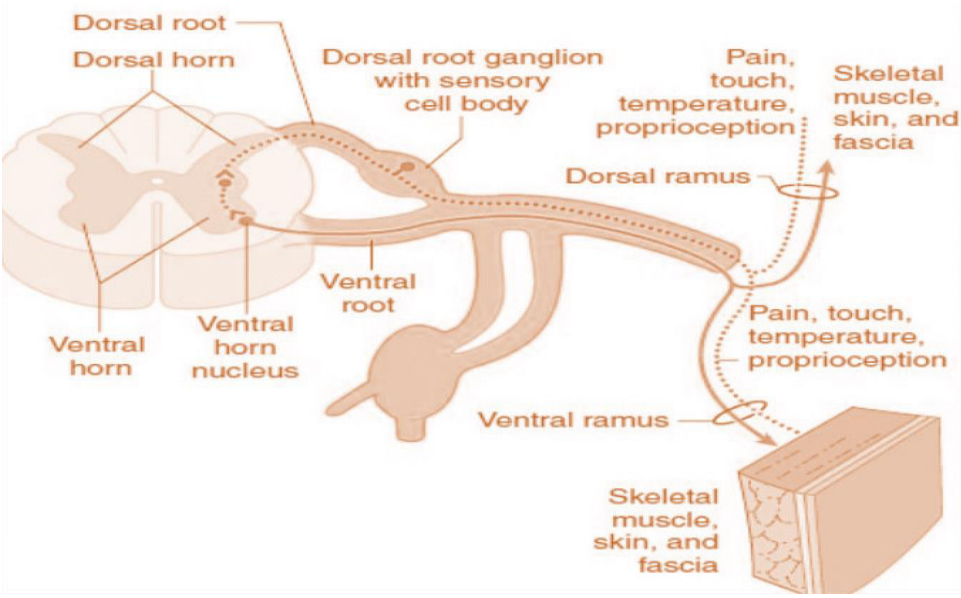


Figure 1: Spinal cord cross-section

2.2 PERIPHERAL NEUROPATHY

Traumatic injury, Hereditary, metabolic, infectious, inflammatory, and toxic chemicals are the most common causes of peripheral neuropathy. Based upon the severity of nerve damage Peripheral neuropathy can be classified as given below.[25]

a: Mononeuropathy

It mainly affects Single nerves or their branches and pain generated at one site frequently in the limbs and then spread to other sites in an asymmetrical pattern. It targets the entire nerve trunk and connects neuronal tissue, including blood vessels.[26]

b: Polyneuropathy

It Impact all peripheral nerves including long fibers of the feet which are primarily affected and damages the axons and influence demyelination.[27]

c: Demyelinating polyneuropathy

Heavily myelinated fibers are mainly impacted by this kind of neuropathy leading to loss of myelin and Schwan cells. It may be acquired or heredity The Guillain Baree syndrome is an example of this type of neuropathy.[28]

d: Axonal Polyneuropathy

It disturbs cellular constituents in axonal microtubules and microfilaments result in axonal dysfunction. Numerous conditions are involved in the generation of distal axonopathy like metabolic; diabetes, nutritional; vitamin B12 deficiency, toxicity; with neurotoxic drugs and alcohol. The sensory symptoms most often start in the distal extremities in a glove-and-stocking distribution. Motor symptoms manifest as distal weakness and muscle atrophy. In addition, autonomic symptoms like; orthostatic hypotension; cardiovascular disturbances; erectile dysfunction or gastrointestinal disturbances are common. [29]

2.3 PAIN

“Pain” word had come from the ancient Greek word "Poine" which means penalty, However, the International Association for the Study of Pain IASP defined Pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.[30] Nociceptors or pain receptors consist of many channels responsible for detecting and transmitting noxious heat, cold, mechanical, and chemical stimuli.[31] Various ion channels are reported to play a vital role in the modulation of nerve conduction and regulation of neurotransmitter release involved in pain transmission.[32] Depending upon its cause and origin pain can be categorized into the following categories;

a. Nociceptive pain:

This type of pain is evoked by high strength noxious stimuli and is mainly mediated by high threshold unmyelinated C fiber or thinly myelinated A-delta primary sensory neurons that feed into nociceptive pathways of the central nervous system (CNS).[33]

b. Inflammatory pain:

This type of pain is evoked due to tissue injury or the consequent inflammatory response generates inflammatory pain. Usually, this type of pain disappears after the resolution of the initial tissue injury. However, in chronic disorders such as rheumatoid arthritis, the pain persists for as long as the inflammation persists. [34]

c. Dysfunctional pain:

This pain is associated with conditions such as fibromyalgia, irritable bowel syndrome, and interstitial cystitis where there is no detectable inflammation or damage to the nervous system is found. This pain appears as a result of an autonomous amplification of nociceptive signals in the CNS with a disturbed balance of excitation and inhibition in central circuits and altered sensory processing.[35]

d. Neuropathic pain:

Various noxious conditions including mechanical trauma, metabolic diseases, neurotoxic chemicals, infection, or tumor invasion and involvement of multiple pathophysiological conditions both within the PNS and in the CNS result in damage of peripheral nervous system which ultimately evokes painful sensation which is known as neuropathic pain.[36] IASP-International Association for the Study of Pain defined it as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". The somatosensory system allows for the perception of touch, pressure, pain, temperature, position, movement, and vibration. The somatosensory nerves arise in the skin, muscles, joints, and fascia and include thermoreceptors, mechanoreceptors, chemoreceptors, preceptors, and nociceptors that send signals to the spinal cord and eventually to the brain for further processing. [37]

2.4 RECEPTORS INVOLVED IN PAIN SIGNALLING

TRPVI (Transient receptor potential vanilloid subtype 1) is a Vanilloid type receptor expressed in the Dorsal root ganglion DRG and senses noxious heat above 43^0 C.[38] After experimental nerve injury, its expression is increased.[39] TRPM8 (Transient receptor potential cation channel subfamily M (melastatin) member 8) is expressed in DRG and is activated by menthol and temperature below 25^0 C.[40] TRPV4, T-type Ca2+ channels, and

two-pore K+ channels and with several candidate receptors are involved in mechano-transduction.[41] TRPA1 (Transient receptor potential cation channel subfamily A1) receptors are inflammatory mediators that respond to cytokines, histamines, prostaglandins, bradykinin, and substance-P.[42] (Table 1)

S. No	TYPE OF STIMULUS	RECEPTOR INVOLVED
1	Heat	TRPVI and TRPV2
2	Cold	TRPM8
3	Mechanical transducers	TRPV4
4	Inflammatory mediators	TRPAI

Table 1: Receptors involved in pain signaling

2.5 PAIN TRANSMISSION

Various types of stimuli including high heat, pressure, or chemicals initiate acute pain transmission by the peripheral nerve terminals of nociceptive neurons. These stimuli activate the Nociceptors and conduct electrical impulses from the nociceptive fiber terminal along the axons to the dorsal horn of the spinal cord.[43] Primary sensory neurons transport the signals from large myelinated Aβ and Aδ fibers and unmyelinated C-fibers. Aβ -fibers respond to light touch and carry non-noxious stimuli, and this information is altered during tissue injury or in diseased conditions. Whereas, mechanical, chemical, and thermal threats are sensed by Aδ and C- fibers. Individual fiber terminates in their distinctive lamina in the dorsal horn of the spinal cord. Aβ -fibers terminate in inner laminae III, IV, and V, and Aδ -fibers terminate in laminae-I and V of the dorsal horn. The C-fibers terminate in inner lamina-I and outer lamina-II.[44] A single axon of sensory neuron splits up into peripheral branch which ranges to nociceptors and central branch which enters to the central nervous system where it synapses with second-order neurons in the dorsal horn of the spinal cord. Peripheral branches of neurons originating from lumbar DRG terminate in the hind paw, and the associated central branches terminate in the medial portion of the dorsal horn.[45] Further second-order pain projection neurons cross contra laterally on the spinal cord and join ascending fibers of the brain stem and brain.[46]

2.6 PERIPHERAL AND CENTRAL MECHANISMS INVOLVED IN NEUROPATHIC PAIN TRANSMISSION

Any injury to the somatosensory system generates nociceptive input which further triggers maladaptive plasticity in the nervous system and results in chronic neuropathic pain.[47] At the site of tissue injury a wide range of inflammatory and signaling molecules such as protons, ATP, PGE2, thromboxanes, leukotrienes, endocannabinoids, NGF, G-CSF, GM-CSF, cytokines (IL6, IL ß, TNF-α), chemokines, neuropeptides; calcitonin gene-related peptide (CGRP), substance-P, bradykinin, histamine, lipids, and diverse proteases are released depicted.[48] These inflammatory mediators are mainly responsible for peripheral sensitization. A concurrent release of such inflammatory mediators in the spinal dorsal horn, in turn, facilitates pain perception by amplifying the nociceptive impulse to the brain. This activity-dependent spinal sensitization results in co-release of glutamate, substance P, calcitonin gene-related peptide (CGRP) all of which are involved in NMDA-Receptor activation further resulting in sustained depolarization of postsynaptic neurons by effluxing Mg^{2+} and influxing of Ca^{2+} from NMDA channels into the neuron.[48] The influx of Ca^{2+} ions facilitates the release of nitric oxide (NO) by activating neuronal nitric oxide synthase and prostaglandins by cyclooxygenase enzymes.[49] These both molecules together enhance the excitability of spinal neurons in response to an incoming pain signal and facilitate the exaggerated release of neurotransmitters from presynaptic sensory neurons towards the spinal cord.[50] High nociceptive input through the multiple receptors like AMPA, NKl (Neurokinin-1), TrkB (Tropomyosin receptor kinase B), mGluR(metabotropic glutamate receptors) expressed in dorsal horn neurons results in the activation of PKC, ERK and p38 alter ion channel kinetics and initiate microglial cells for the releasing BDNF, TNF-α, IL-6, and cathepsin-S for altering neuronal functions.[51] The microglia-derived TNF-α further stimulates the astrocytes for releasing IL-lβ and other related messengers on presynaptic terminals which results in excess release of glutamate for generating increased excitatory postsynaptic potential (EPSP) via TRPVI channel activation.[52] The increased glutaminergic and decreased GABAergic transmission in the dorsal horn seems to be important in increasing pain perception.[53] Overall peripheral and central mechanism involved in the transmission pain and sensation is summarized in Figure: 2

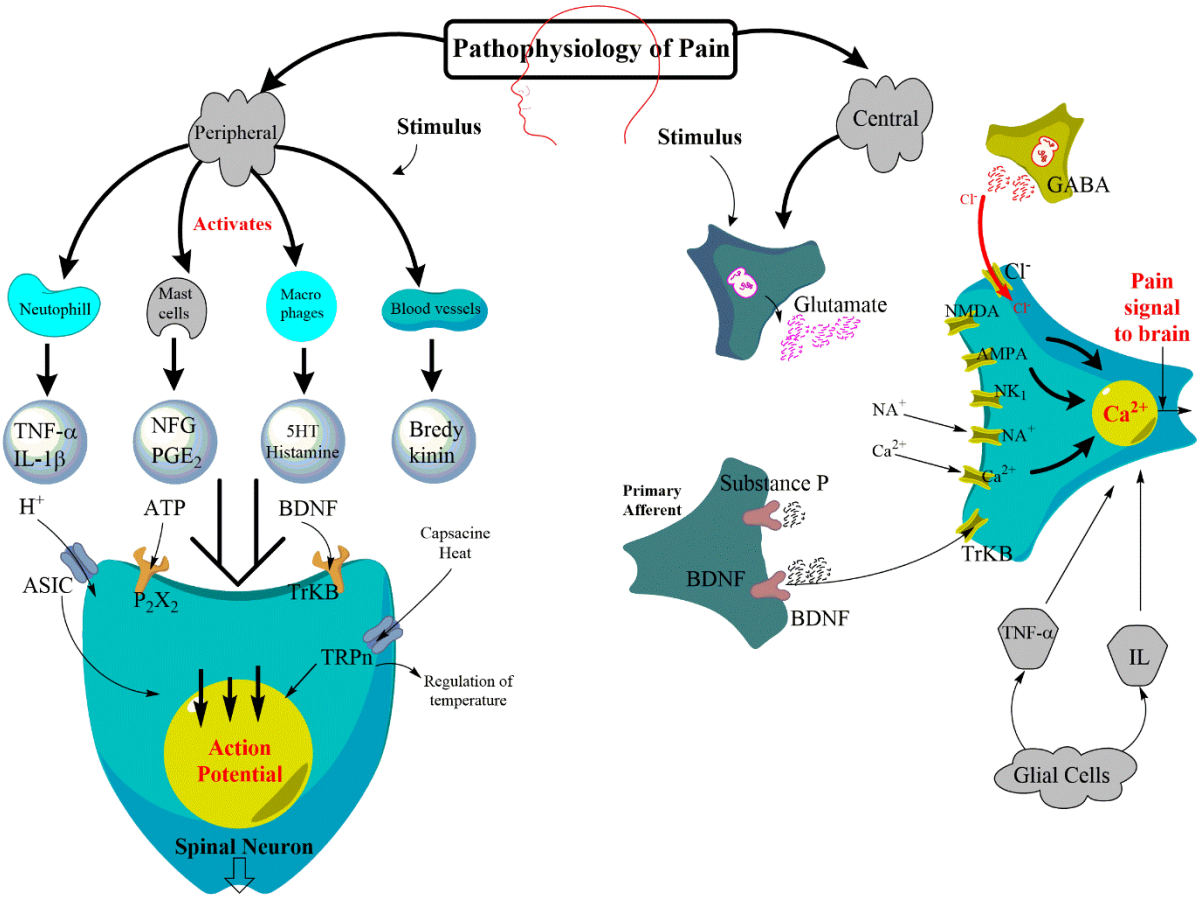


Figure 2: Mechanisms involved in neuropathic pain transmission

Notes: (A) Peripheral sensitization and (B) central sensitization. Activation of peripheral nociceptors on the skin in response to stimuli, such as heat, injury or mechanical pressure, initiates the release of chemical mediators at the site of injury (peripheral sensitization). Persistent pain or inflammation causes activation and repetitive firing in afferent C-fiber nociceptors, which triggers the release of excitatory neurotransmitter glutamate in the synapse of the dorsal horn (central sensitization). This is accompanied by the release of substance P, BDNF and neurokinins, which causes persistent depolarization of the cell membrane. Additionally, activation of AMPA or NMDA receptors by glutamate stimulates the microglia and subsequently induces the release of cyclooxygenase enzymes 1 and 2, nitric oxide and other proinflammatory mediators (TNF-α, IL-1, IL-6). GABA is secreted by interneurons of descending inhibitory serotonin pathway from brain and stops pain signal by activating Cl^- channels on neurons.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; ASIC, acid-sensing ion channels; BDNF, brain derived neurotropic factor; 5-HT, 5-hydroxytryptamine; IL, interleukin; NMDA, N-methyl-D-aspartate receptor; NGF, nerve growth factor; PG, prostaglandin; NK1, neurokinin-1; TNF, tumor necrosis factor; TrkB, tyrosine receptor kinase B; TRPV, transient receptor potential vanilloid receptor. Gamma-aminobutyric acid (GABA).

2.7 SIGNS AND SYMPTOMS OF NEUROPATHIC PAIN

Basic characteristics of neuropathic pain; include pain arising without stimulus or abnormal response to non-painful or painful stimuli. Patients may complain about dysesthesia; tingling, pins and needles, and deep-seated gnawing pain with burning shooting stabbing, with sharp electric shock symptoms. Features of neuropathic pain may come out within days of nerve damage or can take months to develop.

Symptomatic Pain terms (As defined by the International Association for the Study of Pain)[54]

- **Allodynia:** Pain due to a stimulus that does not normally provoke pain
- **Analgesia:** Absence of pain in response to stimulation that would normally be painful **Hyperalgesia**: An increased response to a stimulus that is normally painful
- **Hyperesthesia:** Increased sensitivity to stimulation, excluding the special senses
- **Hyperpathia**: A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold
- **Hypoalgesia:** Diminished pain in response to a normally painful stimulus
- **Hypoesthesia**: Decreased sensitivity to stimulation, excluding the special senses

2.8 NEUROPATHIC PAIN INDUCTION TECHNIQUES IN RODENTS

Animal models of neuropathic pain generate similar symptoms as those generated in humans. Hence, disparate neuropathic pain, evaluating techniques has been implemented. Numerous neuropathic induction techniques are established in animal models for evaluating and understanding the mechanism of neuropathic pain for developing an effective therapy for its optimal management. Various Experimental and drug-induced models for neuropathic pain are enlisted below

A) Chronic nerve injury models.[55]

- Complete sciatic nerve transaction
- Chronic constriction injury
- Spinal nerve ligation
- Ligation of common peroneal nerve Ligation of common peroneal nerve
- Excitotoxic spinal cord injury
- Weight drops or contusive spinal cord injury
- Spinal hemisection

B) Cancer-induced pain model.[56]

- Femur, calcaneus, tibial, humerus bone cancer pain, etc.,

C) Anti-HIV drug-induced neuropathy[57]

- Zalcitabine

D) Viral disease-induced neuropathy.[58]

- Varicella-Zoster

E) Disease-induced nerve injury model.[59]

- Streptozotocin-induced

F) Drug-induced neuropathy.[60]

- Anti-cancer agents (vincristine, cisplatin, oxaliplatin, paclitaxel).

The generating symptoms like tingling, burning pain in the arms, allodynia, and hyperalgesia are the same in Chronic nerve injury models and other disease or drug-induced neuropathic pain models.[61] In the above-mentioned techniques; Chronic nerve injury models specifically the CCI-Chronic constriction injury model are one of the most reliable models of neuropathic pain hence various researchers follow this method for evaluating the potential of compounds against neuropathic pain. This surgical technique has accurate symptomatic outcomes and the development of neuropathy take place in a short duration of time.

Chronic constriction injury (CCI) to sciatic nerve:

Chronic constriction injury simulates the symptoms of chronic nerve compression in clinical conditions of trauma or tumor developments. This model also mimics a lesion of nerve fibers located mainly at the surface of the peripheral nerve (a typical feature of, for example, carpal tunnel syndrome).[55]

Procedure: In the CCI model, a 1.5-cm incision is made 0.5 cm below the pelvis. The biceps femoris and the gluteus superficial are separated and the sciatic nerve is exposed and isolated and four loose ligatures (5-0 chromic catgut) with 1-mm spacing are placed around it. CCI also

results in the development of allodynia-like behavior, hyperalgesia, and spontaneous pain like behaviors, which normally reach a maximum of 10-14 days after surgery. In this model, chromic catgut contributes to the inflammatory component in addition to neuropathic pain.[55] (Figure 3)

Merits of CCI Model

1. It is a reliable and easily reproducible model
2. It is a frequently used model, particularly for behavioral studies.
3. It results in intraneural edema, which strangulates the nerve, effectively axotomizing many but not all of the nerve axons
4. It mimics the post-traumatic peripheral painful neuropathy states in humans.
5. Partial denervation preserves some behavioral responses to peripheral stimuli.
6. It shows behavioral signs of spontaneous pain.
7. This model produces a greater reduction (almost all Aβ fibers) and a very large majority of as fibers are axotomized while large numbers of C-fibres are intact.
8. This model involves both inflammatory components and neuropathic components.
9. This is a suitable model for assessing cold allodynia when compared to partial sciatic nerve ligation (PNL) and spinal nerve ligation (SNL).
10. This model involves both neuropathic and inflammatory components

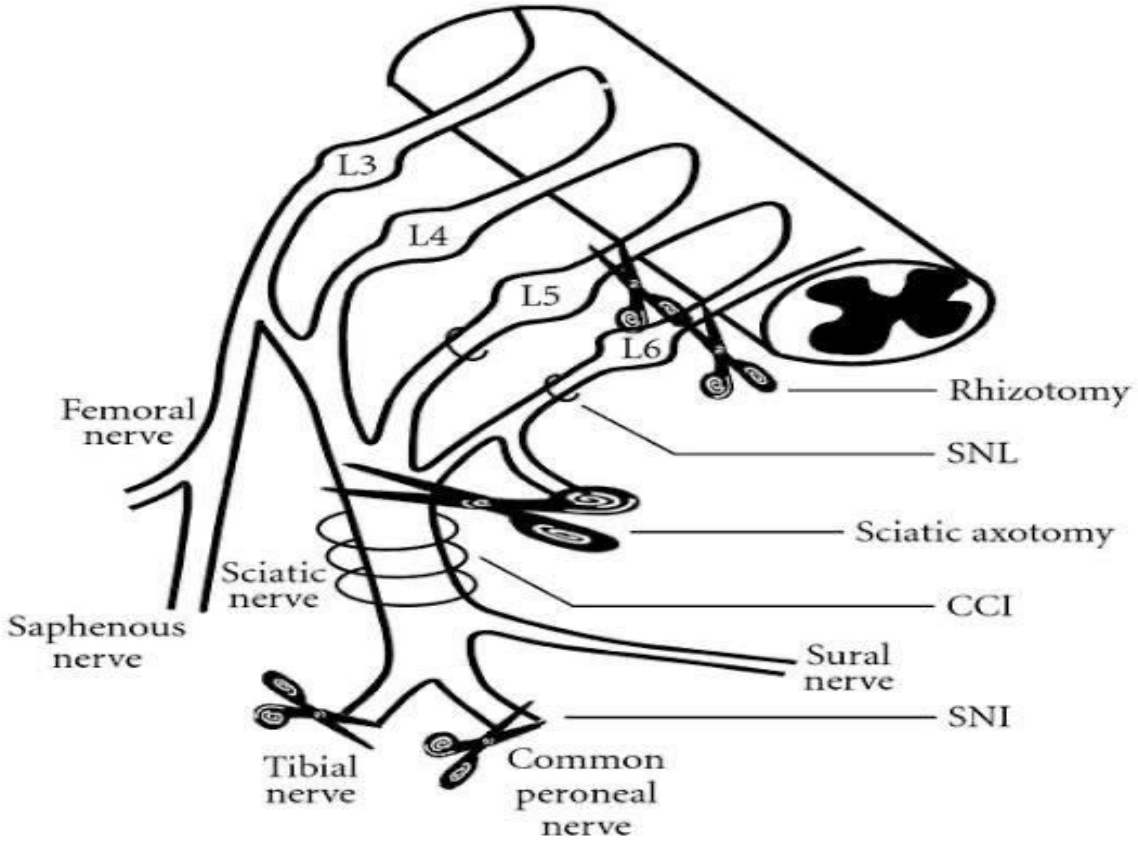


Figure 3: Chronic nerve injury models

2.9 AVAILABLE PHARMACOTHERAPY FOR NEUROPATHIC PAIN. (Table 2) [5]

Drug	Mechanisms of action	Adverse effects	Contraindications
Tricyclic antidepressants			
Nortriptyline, desipramine, amitriptyline, clomipramine imipramine	Monoamine reuptake inhibition, sodium channel blockade, and anticholinergic effects	Somnolence, anticholinergic effects and weight gain	• Cardiac disease, glaucoma, prostatic adenoma, and seizure High doses should be avoided in adults >65 years of age and those with amyloidosis
Serotonin-noradrenaline reuptake inhibitors			
Duloxetine	Serotonin and noradrenaline reuptake inhibition	Nausea, abdominal pain, and constipation	Hepatic disorder and hypertension• Use of tramadol
Venlafaxine	Serotonin and noradrenaline reuptake inhibition	Nausea and hypertension at high doses	Cardiac disease and hypertension• Use of tramadol
Calcium channel α2δ ligands			
Gabapentin, extended-released gabapentin and enacarbil, and pregabalin	Act on the α2δ subunit of voltage-gated calcium channels, which decrease central sensitization	Sedation, dizziness, peripheral edema, and weight gain	Reduce dose in patients with renal insufficiency
Topical lidocaine			
Lidocaine 5% plaster	Sodium channel blockade	Local erythema, itching, and rash	None
Capsaicin high-concentration patch (8%)	Transient receptor potential cation channel subfamily V member 1 agonist	Pain, erythema, itching, and rare cases of high blood pressure (initial increase in pain)	No overall impairment of sensory evaluation after repeated applications and caution should be taken in progressive neuropathy
Opioids			
Tramadol	μ-Receptor agonist and monoamine reuptake inhibition	Nausea, vomiting, constipation, dizziness, and somnolence	History of substance abuse, suicide risk and use of the antidepressant in elderly patients
Morphine and oxycodone	μ-Opioid receptor agonists; oxycodone might also cause κ-opioid receptor antagonism	Nausea, vomiting, constipation, dizziness, and somnolence	History of substance abuse, suicide risk, and risk of misuse in the long term
Neurotoxin			
Botulinum toxin A	Acetylcholine release inhibitor and neuromuscular-blocking agent; potential effects on mechanotransduction and central effects in neuropathic pain*	Pain at the injection site	Known hypersensitivity and infection of the painful area

Table 2: Available pharmacotherapy for neuropathic pain.

The data shows the potency and efficacy of Available pharmacotherapies for neuropathic pain. However due to their significant adverse effects and other contraindications, nowadays researchers are diverted towards herbal plants for their pharmacological potential in attenuating neuropathic pain.

2.10 IMPORTANCE OF MEDICINAL HERBS IN NEUROPATHIC PAIN

Many novel phytoconstituents are obtained from plant sources of the traditional medicinal systems and play a vital role in curing various life-threatening diseases. India being a rich source of medicinal plants is well known as the "Botanical Garden of the world". Approximately 2500 plant species are used in the Indian traditional medicinal system.[62, 63] 80% of the world population still rely on traditional medicinal systems for curing disease.[64] Various phytochemical constituents from different herbal plants Including phenols, alkaloids, flavonoids, terpenoids, coumarins, and glycosides had shown positive effects in the treatment of neurological disorders including neuropathic pain.[64]

Kassuya CA et al. reported Anti-allodynic properties of the hexanic extract of Phyllanthus ***amarus*** in models of persistent neuropathic pain possibly by increasing myeloperoxidase activity.[65]

Comelli F et al. 2008 reported the Antihyperalgesic effect of a Cannabis sativa extract in a rat model of neuropathic pain by acting on vanilloid receptors TRPV1.[66]

Thiagarajan VR et al. 2012 had shown that ethanolic extract from leaves of ***Butea monosperma*** on pre-treatment of rats attenuates neuropathic pain in the CCI-induced neuropathic pain model. The effect of the plant is proposed due to its anti-oxidative and calcium channel modulatory potentials.[67]

Moini Zanjani T et al. 2014 reported that ***Curcuma longa*** reduced mechanical and cold allodynia and attenuated the serum concentration of cyclooxygenase 2 (COX-2) in the CCI model of neuropathic pain in rats possibly by reducing the inflammatory effects of COX-2 enzyme activity.[68]

Diwane C et al. 2015 reported the potential of alcoholic extract of roots and rhizomes of ***Rubia cordifolia*** in significantly decreasing withdrawal latency in cold allodynia method and withdrawal latency in the hot plate method in paclitaxel-induced neuropathic pain in rats possibly by acting o GABA or antioxidant mechanism.[69]

Zhao WX et al. 2017, demonstrated that **diosgenin** (a steroidal saponin extracted from numerous plants, including Solanum and Dioscorea species) attenuates neuropathic pain in CCI rats by inhibiting activation of the p38 MAPK and NF-κB signaling pathways.[70]

Singh et al. 2017 showed that methanol extract of ***Alstonia scholaris*** significantly attenuated heat hyperalgesia, mechanical hyperalgesia, and cold allodynia as well as protection against oxidative stress and inflammatory activity in CCI rats probably due to the presence of kaempferol and attributed to inhibit the inflammatory cytokines and ROS production.[71]

Ghasemzadeh Rahbardar M et al. 2017 reported anti-inflammatory effects of ethanolic extract of ***Rosmarinus officinalis*** by decreasing spinal inflammatory markers comprising matrix metallopeptidase 2 (MMP2), COX2, IL-1b, and PGE- 2 in CCI rats. [72]

Borgonetti V et al., 2020 Showed that oral administration of ***Zingiber officinale*** Roscoe rhizomes extract (ZOE) obtained by CO2 supercritical extraction attenuated SNI-induced neuropathic pain symptoms by reducing spinal neuroinflammation.[73]

Khan D. et al. 2020 Reported neuroprotective effects of the ***Allium cepa leaves*** extract on Diabetes Induced and Chronic Constriction Injury Induced Neuropathic Pain in Experimental Rats is possibly by its strong antioxidant activity.[74]

Amita JT. et al. 2020 Showed that extract of W***ithania somnifera*** attenuates tibial and sural transection induced neuropathic pain possibly by its inhibitory action on myeloperoxidase and TNF-α.[75]

Verma S. et al. 2020 Reported neuroprotective effect of ethanolic extract of ***Solanum virginianum Linn***. (SV) in chronic construction injury (CCI) of the sciatic nerve–induced neuropathic pain possibly by restoring antioxidant level and pro-inflammatory cytokine (IL-1β and TNF-α) expression in a dose-dependent manner and modulating of calcium and sodium channels.[76]

Bomba FD. et al. 2021 Showed that methanolic extract of stem bark of ***Petersianthus macrocarpus*** possess a potent anti-hypernociceptive effect on CCI neuropathic pain possibly by inhibition of the nitric oxide pathway as well as the reduction in NF-κB and TNF-α gene expression in the brain may at least partially contribute to this effect. [77]

Kroth A et al. 2021 showed that Aqueous extract from ***Luehea divaricata*** leaves improves SFI and modulate oxidative stress markers in the injured sciatic nerve and spinal cord of CCI rats. They suggest that the antinociceptive effect of the extract involves modulation of oxidative stress markers in the injured sciatic nerve and spinal cord.[78]

Rakhshandeh H et al. 2021 showed the neuroprotective potential of hydroalcoholic extract of ***Lawsonia inermis*** on neuropathic pain induced by chronic constriction injury possibly due to its antioxidant and anti-inflammatory effects.

Various Herbal medicines are also investigated in clinical trial as enlisted in Table:3[17]

SUBSTANCE	NEUROPATHIC DISORDERS	STUDY TYPE	RESULTS
A9-Tetrahydrocannabinol /Cannabidiol (THC/CBD)	HIV-associated distal sensory predominant polyneuropathy	Phase II, double-blind, placebo-controlled, crossover trial	Reduced neuropathic pain intensity
THC/CBD (tetrahydro cannabinoid)	Central neuropathic pain in patients with multiple sclerosis	Randomized controlled trials	Improvement in neuropathic pain without evidence of tolerance
THC (tetrahydro cannabinoid)	Patients with terminal cancer related pain refractory to Strong opioid	An open-label extension study	Well tolerated and reduced pain
THC/CBD (tetrahydro cannabinoid)	Patients with peripheral neuropathic pain	A double-blind, randomized, placebo-controlled, parallel group study	Improvement in neuropathic pain
THC/CBD (tetrahydro cannabinoid)	Patients with peripheral neuropathic pain	A multicentre, open-label, follow-on study	Improvement in neuropathic pain
Aconitum	Patients with diabetic peripheral neuropathic pain	Controlled clinical trials study	Reduced diabetic peripheral neuropathic pain
Citrullus colocynthis	Painful diabetic polyneuropathy patients	Double-blind randomized placebo-controlled clinical trial	Reduced diabetic polyneuropathy pain
Goshajinkigan	Oxaliplatin-induced neuropathy patients	Phase 2, multicenter, randomized, double-blind, placebo-controlled trial	Delayed the onset of grade 2 or greater oxaliplatin-induced neuropathy

Table:3 Herbal medicines and their constituents evaluated in clinical trial

The above-reviewed literature shows the potential of herbal plants in attenuating neuropathic pain without any significant adverse effects. The present chosen plant *Viola odorata L. Linn.* has been traditionally used for treating pain. Paste of fresh leaves of ***Viola odorata L.*** is applied locally to relieve pain and inflammation.[19] Besides its, Viola odorata L. has also been reported to contain a variety of phytochemicals that can play a vital role in alleviating neuropathic pain.[20] However, due to the lack of scientific data on the potential of *Viola odorata L.* in attenuating CCI (chronic constriction injury) induced neuropathic pain, there is a need for further studies for the same.

2.11 *VIOLA ODORATA L. LINN*

Description

Viola odorata L. Linn. belongs to the family Violaceae. It is commonly known as Sweet Violet, English Violet, Common Violet, or Garden Violet, and Gulbanafsa in Hindi. It is a perennial stock short, but sometimes branched, knotted, with the remains of the old leaf-stalks and stipules, and usually emitting creeping runners or scions. The plant blooms in continental climate conditions in late winter or early spring (March-April) with delicate flowers of attractive scent because of which it is frequently gathered from its natural habitats. It is a glabrous or pubescent herb, about 15cm in height. Leaves are dark green, tough, broadly ovate, or cordate in shape with a crenate margin. They are 1.5 to 5 cm in size. Flowers are solitary, auxiliary forming central flowering rosettes, and are deep violet in shade with bluish-white base, sweet, scented and hence plant is cultivated in gardens as an ornamental crop. Fruits are in the form of capsules, round, three angled, and often purplish in color.[80]

Figure 4: Viola odorata L. l Plant

Scientific classification[21]

Phylum: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Violates

Family: Violaceae

Genus: Viola

Species: V. odorata Linn

Vernaculars name[80]

Arabic: Banafsaj and Firfeer

English: Sweet violet and wild violet

Hindi: Banafshah

Sanskrit: Banaphsha, Nilapushpa

Urdu: Gule Banafsha

Kannada: Vialtethoo

Tamil: vialtethoo and Vilaettu

Bengali: Banafshah and Banosa

Geographical distribution

V. odorata is a native of Mediterranean countries and Asia Minor. From old ages, it has been grown in gardens, and now it has spread to most of Europe. It is found in high altitudes of the Himalayas, Europe, and throughout North America. the plant has also been found widely in the northern areas of Iran, as well. Sweet violet (*Viola odorata L.*) Grows wild, in places exposed to sun, alongside hedges, riverbanks, on the edges of deciduous forests, and in forest glades. It is widespread all across Croatia, along with the mentioned species, lists further 19 species of the same genus. The Mediterranean is considered to be the sweet violet's original habitat, and nowadays it ranges from the North of Europe to South Africa, Tierra del Fuego, and Australia. V. odorata is indigenous to India and found in Kashmir, Himachal Pradesh, and Kumaon hills. It is distributed in Kashmir and the western Himalayan regions at an altitude of 1500 to 1800 m.[81-83]

Traditional uses

V. odorata is commonly used as a remedy for coughs and sore throat, hoarseness, and tonsillitis. The herb is valued as an expectorant, diaphoretic, antipyretic, diuretic, and as a laxative, in bilious affections. Paste of fresh leaves of *Viola odorata L.* is applied locally to relieve pain and inflammation.[19] The scent of violet flowers is distinctive with only a few other flowers having a remotely similar odor. V. odorata is used either alone or in a mixture with other herbs for catarrhal and pulmonary troubles and calculous affections. The pharmacological study revealed the role of V. odorata in some Unani drugs for the treatment of common cold, asthma, coughs, and fevers.[80]

2.12 PHYTOCHEMICAL COMPOSITIONS OF *VIOLA ODORTA L.*

The flowers of V. odorata is mainly contained odorant principles, blue coloring matter, and sugar. Viola-quercetin is found in all parts of the plant. Salicylic acid (natural aspirin) has also been yielded from this medicinal herb. The phytochemical screening of different extracts including n-hexane, butanol, methanol, and aqueous of V. odorata has shown that plant contains flavonoids, glycosides, alkaloids, steroids, terpenes, saponins, and tannins which are very important constituents when looking for pharmacologically active phytochemicals.[84] Enzyme-linked immunosorbent assay (ELIZA) method on quantification of 50% methanol extract of violet flower shows the presence of melatonin in Viola odorata L. High percentages of monoterpenes and sesquiterpene phenyl butanone, linalool, benzyl alcohol, α-cadinol, globulol, and viridiflorol are obtained from the essential oil of violet flowers.[85] Further Essential oil of violet leaves is reported to contain butyl-2-ethyl hexyl phthalate and 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone.[86]. The methanolic leaves extract of V. odorata was found to have a total of 34.4 mg/g phenolic and 22.8 mg/g flavonoid contents.[87] An alkaloid violine is found in roots, leaves, flowers, and seeds of V. odorata. Further few studies had also reported the presence of saponins, cardiac glycoside, methyl salicylate, mucilage, vitamins A and C, and alkaloids in the essential oil of V. odorata.[88] Flowers of V. odorata contain 4.0 % anthocyanins, 1.1 % flavonoids, 0.4 % glycoside, 18.0 %mucilage and 8.5% ash. Studies also identified various serotonergic flavonoids including 5,7-Dihydroxy-3,6-dimethoxyflavone, 5,7,4′-trihydroxy-3′,5’dimethoxyflavone, and 5,7,4′-trihydroxy-3′-methoxy flavone in areal parts of Viola odorata L. Studies also showed the presence of coumarins and terpenes in the leave extract of Viola odorata L. Former pharmacological

studies revealed the role of V. odorata in some Unani drugs for the treatment of common cold, asthma, cough, and associated ailments[91].

2.13 PHARMACOLOGICAL ACTIVITY OF *VIOLA ODORATA L*:

Khattak et al. 1985 reported that *V. odorata* produced a significant oral ***antipyretic activity*** in rabbits using hexane, chloroform, and water-soluble extracts. The antipyretic activity was more prominent in the hexane-soluble portions of V. odorata.[92]

Koscheck et al. 2003 reported the aqueous extract of V. odorata showed ***anti-inflammatory*** properties as compared with hydrocortisone. V. odorata extract given prophylactically was partially effective in preventing lung damage, equal to the effect of hydrocortisone in aiding the resolution of formalin-induced lung damage.[93]

Vishala A et al. 2009 reported that alcoholic extracts at a dose level of 200 mg/kg and aqueous extract of V. odorata at a dose level of 400 mg/kg showed significant effect as ***laxatives.***[84]

Vishal A., et al. 2009 investigated the aerial part of *Viola odorata L.* for various biological activities. The butanolic, aqueous, and n-hexane fraction of the aerial parts of *Viola odorata L.* exhibited a good ***diuretic*** effect in rats.[84]

Gerlach et al. 2010 reported that the cycloviolacin O2, a cyclotide from V. odorata showed ***antitumor activity*** and causes cell death by membrane permeabilization.[94]

Ebrahimzadeh M.A., et al. 2010 investigated that the crude methanolic extract of *Viola odorata L.* has ***antioxidant*** potential against DPPH, reducing power assay, ferric thiocyanate, nitrous, hydrogen peroxide scavenging protocols.[95]

Khan et al. 2011 reported that aqueous extract of V. odorata (flowers) showed strong ***antibacterial action*** against B. subtilis, E. coli, and S. aureus.[96]

Antil V. et al. 2011 reported that crude methanolic extract at the dose of 400 mg/kg of Viola odorata L. was proved **analgesic** in acetic acid-induced writhing and tail immersion animal models.[97]

Ramezani et al. 2012 reported the ***antibacterial activity*** of aqueous extracts of different parts of V. odorata against S. aureus, E. coli, and P. aeruginosa and concluded its maximum effect on S. aureus and minimum effect on P. aeruginosa.[98]

Siddiqi HS et al. 2012 reported that V. odorata shows a reduction in body weight and ***antidyslipidemic effect*** which may be due to the inhibition of synthesis and absorption of lipids and antioxidant activities.[99]

Siddiq H.S. et al. 2012 showed the ***antihypertensive*** effect of leave extract of Viola odorata L. possibly mediated by vasodilator effect of extract through multiple pathways like inhibition of Ca++ influx via membranous Ca++ channels, its release from intracellular stores, and NO-mediated pathways.[99]

Siddiq H.S. et al. 2012 investigated the plant extract of *Viola odorata L.* in anesthetized rats showed a **reduction in body weight** suggested the effect which may be due to the inhibition of synthesis and absorption of lipids and antioxidant activities.[99]

Monadi A., et al. 2013 demonstrated that the injection of the ethanolic and chloroform extracts of *V. odorata* was found to increase sleeping time and also showed better ***sedation and pre-anesthetic effects*** in a dose-dependent manner when compared with diazepam.[100]

Elhassaneen Y et al. 2013 reported that **the** Supplementation of the animal diets with sweet violet blossoms powder SVBP (0.2 to 1.6 g/100g) prevented significantly ($p \leq 0.05$) the rise of mean serum ***AST, ALT, and ALP activities***; urea, creatinine, and MDA levels.[101]

Feyzabadi Z., et al. 2014 showed that intranasal drop of *Viola odorata L.* in each nostril at night before sleeping for one month showed that improvements in sleep and ***Insomnia*** Severity Index scores were significantly ($P < 0.05$) reduced after a month.[102]

Hassan F. et al. 2014 reported that Viola odorata L. contained very active compounds against ***M. tuberculosis H37Rv and M. avium*** which can be used as lead for potential anti-TB drug formulations on further clinical trials.[103]

Helli S., et al. 2016 reported that Viola *odorata L.* extract has dose-dependent inhibitory effects on the development of tongue-induced **dysplasia**.[104]

Azari Z. et al. 2018 reported the ***antidiabetic*** potential of Viola odorata L. Studies showed that 400 mg/kg dose of the aqueous extract of Viola odorata L. and all users (100, 200, and 400 mg/kg) doses of the hydro-alcoholic extract significantly decreased serum glucose levels in the diabetic rats.[105]

Shafei Z. et al. 2018 showed that the acute doses of the hydroalcoholic extract of *Viola odorata L.* were similar to fluoxetine to cause ***antidepressant*** effects possibly by increasing serotonergic activity.[106]

Azari Z. et al. 2020 showed the ***hepatoprotective potential*** of Viola odorata L. Extracts of the plant were shown to improve the liver tissue in terms of Kupffer cell count, inflammation, and congestion.[105]

Uzma Saleem et al. 2021 showed that hydro-methanolic extract of Viola odorata L. possesses memory-enhancing activity possibly by decreased levels of inflammatory

biomarkers, that is, tumor necrosis factor-alpha, nuclear factor-kappa light-chain enhancer of activated β-cells, and cyclo-oxygenase and may prove in the management of ***Alzheimer disease.***[107]

2.14 ANTINOCICEPTIVE POTENTIALS OF BIOACTIVE COMPOUNDS OF VIOLA ODORATA L. (Figure 5)

- Various phytoconstituents including benzyl alcohol, eugenol, linalool, and α-cadinol are found in the essential oil of Viola odorata L. However separately these constituent was reported to have analgesic and antioxidant activities.[108]
- Flavonoids including 5,7-dihydroxy 3,6-dimethoxyflavon, 5,7,4-trihydroxy 3,5-dimethoxyflavon, and 5,7,4-trihydroxy 3-methoxyflavon are found in Viola odorata L. and was reported to increase serotonin level and activity in CNS.[89] Further flavonoids are also reported to have modulatory action on GABA receptors.[109] These actions of flavonoids may help Viola odorata L. to attenuate neuropathic pain by modulation of GABAergic and serotonergic pathways
- Alkaloids and steroids present in the extract of Viola odorata L. are separately shown an antinociceptive effect.
- Coumarins which is found in Viola odorata L. [110] and separately it was reported to have analgesic.[111]
- Aqueous preparations of Viola odorata L. L. Flowering reported to have the presence of anthocyanins.[112] However, anthocyanin separately was proved to relieve CCI-induced neuropathic pain.[113]
- Salicylic acid (natural aspirin) has also been yielded from this medicinal herb which was separately reported to have analgesic activity by inhibiting inflammatory mediators including TNF-α and NFkB thru suppression of cyclooxygenase.[88, 114]
- Melatonin was also found in a 50% methanol extract of flower of Viola odorata L. However, melatonin was reported to increase endogenous opioids and also reported to decrease pain by acting on its receptors.[116] Hence melatonin shall be one constituent of Viola odorata L. for alleviating neuropathic pain.
- The Viola odorata L. exhibited Ca2+ channel blocking activity in isolated rabbit tissue preparations (i.e., jejunum, trachea, and aorta) possibly due to the presence of alkaloids. This calcium channel blocking activity may trigger the analgesic activity of Viola odorata L. against CCI-induced neuropathic pain.[117]

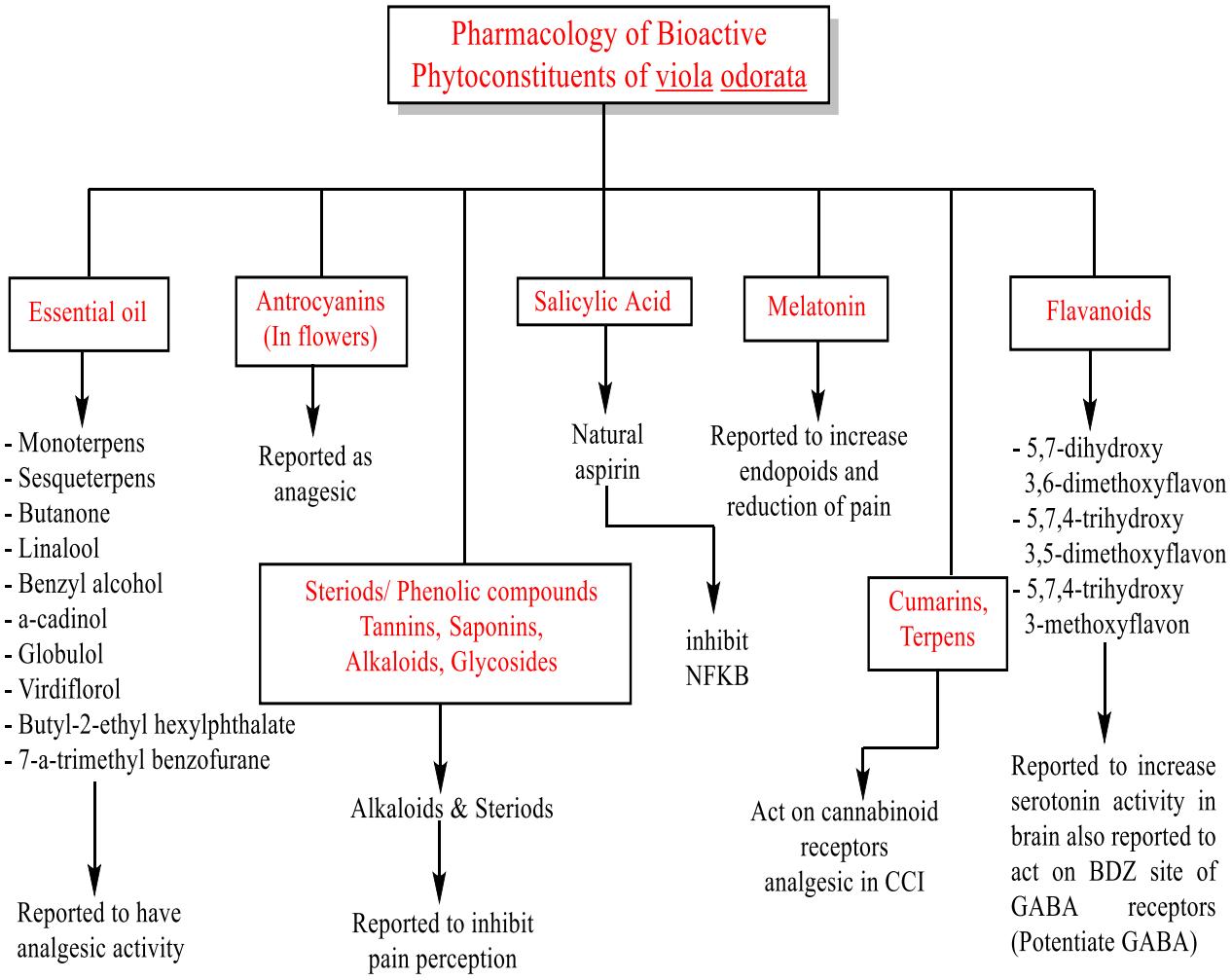


Figure 5: Pharmacological potential of phytoconstituents present in Viola odorata L.

CHAPTER 3: AIM

Till now, no experimental studies have been reported for the potential of Viola odorata L. Linn., in attenuating experimental (CCI) induced neuropathic pain. Hence, the present study is to investigate and validate the ethnical medicinal uses of the plant for its neuroprotective and neuropathic pain-relieving activity.

OBJECTIVES

The following study will be carried out to extract phytoconstituents from aerial parts of viola adorate using different solvents (n-hexane, butanolic, methanolic, and aqueous) The extract obtained will be used to validate its neuroprotective and neuropathic pain-relieving activity in CCI-Chronic constriction injury-induced neuropathic pain in rats.

In this view, the following objectives will be taken into consideration.

1. Pharmacognostic studies on aerial parts of viola adorate
2. Extraction of phytochemicals from aerial parts of viola adorate using Soxhlet apparatus by extracting it continuously for 72 h.
3. Phytochemical investigations on the extracted phytochemical compound (Preliminary phytochemical screening & spectral studies).
4. Pharmacological Screening of viola adorate in attenuating CCI-Induced neuropathic Pain using physical, Behavioral, and biochemical methods.
5. Investigation of Neuroprotective activity of extracted phytochemicals

CHAPTER 4: HYPOTHESIS

Hypothetical Pharmacological potential of *viola odorata L.* is summarized in figure 6

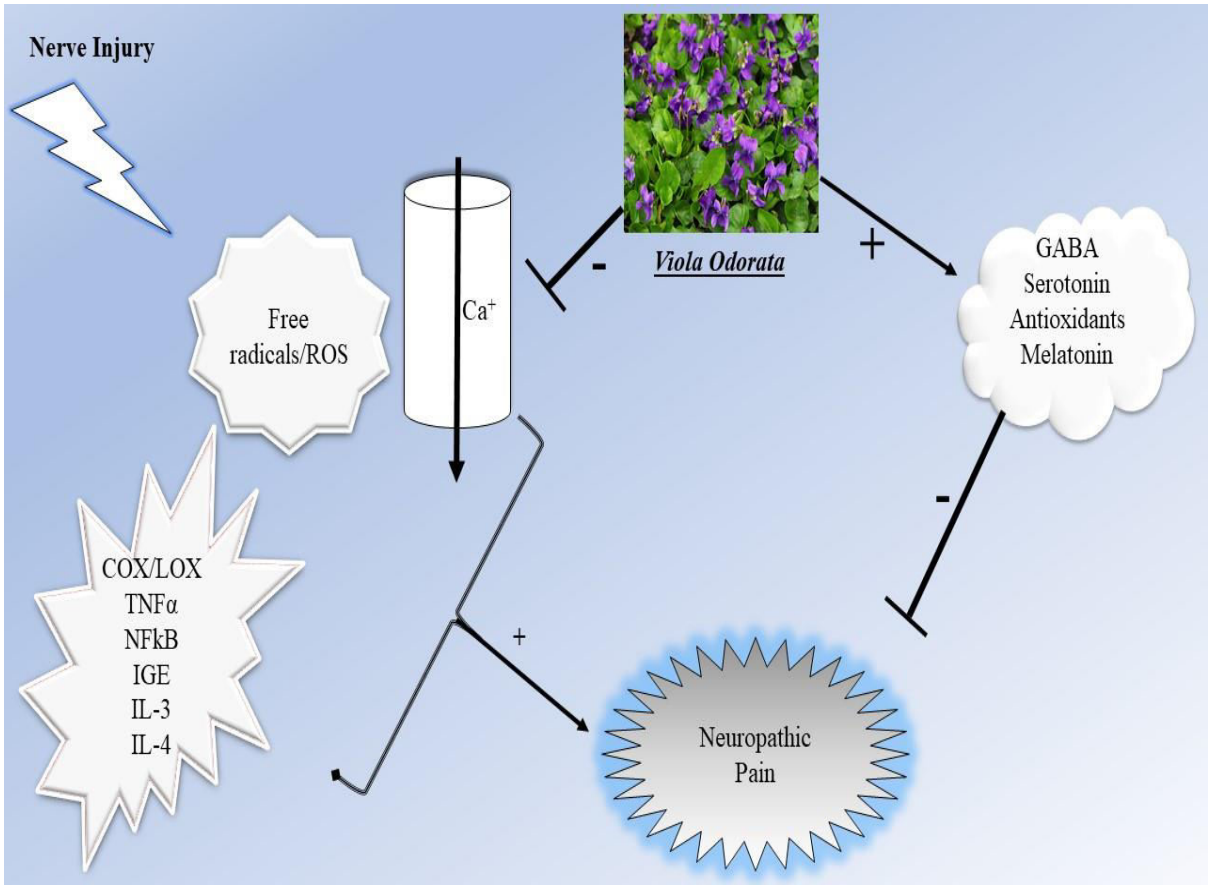


Figure 6: Hypothetical mechanism of action of Viola odorata L. for attenuating neuropathic pain

CHAPTER 5: PLAN OF WORK

Collection of plants from shikari Devi wildlife century tehsil Chachyot district Mandi HP India

↓

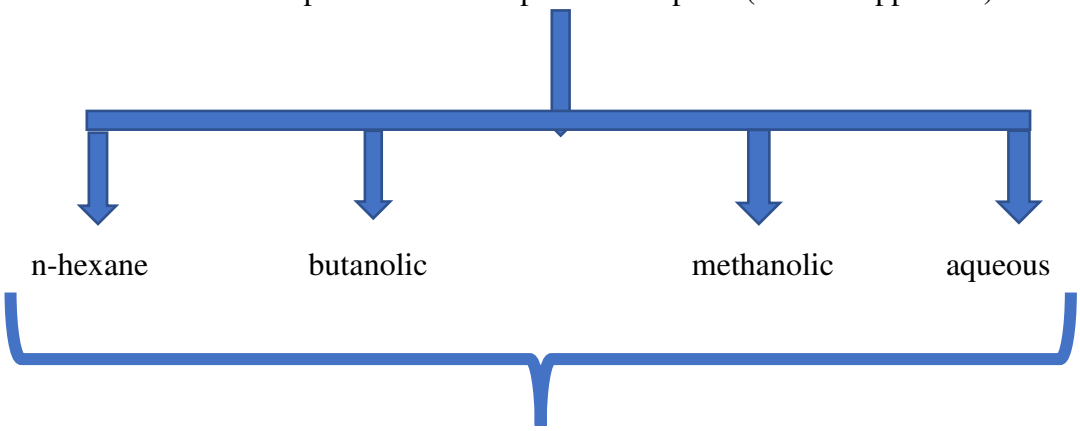
Identification of plant from CSKHPKV agriculture university Palampur District Kangra HP India

↓

Pulverization of dried aerial parts of the plant

↓

Extraction of pulverized dried parts of the plant (Soxhlet apparatus)



Phytochemical screening of bioactive constituents

↓

Pharmacological investigation of extracted phytochemicals

(CCI-induced Neuropathic pain Behavioral and biochemical test)

↓

Compilation and statistical analysis of data

CHAPTER 6: PROPOSED RESEARCH METHODOLOGY

6.1: PHARMACOGNOSTIC STUDIES:

A: Collection, authentication, preparation, and storage of plant material

Plant collection

The Herbal plant Viola odorata L. will be collected from Shikari Devi wildlife century tehsil Chachyot district Mandi HP India.

Authentication

Authentication will be done from CSKHPKV agriculture university Palampur District Kangra HP India

Preparation and storage

The dried areal parts of the herb will be pulverized with a mechanical grinder will be kept in an airtight container up to further use.

B: Extraction of Phytochemicals from Viola odorata L.

Extraction of the pulverized dried aerial part of V. odorata will be done by Soxhlet apparatus using different solvents including n-hexane, butanolic, methanolic, and aqueous.[97]

C: Phytochemical investigations on Viola odorata L.

All the four extracts (i.e., n-hexane, butanolic, methanolic, and aqueous) of V. odorata will be subjected to the preliminary phytochemical screening as per standard procedures. [118, 119]

- Determination of Alkaloids
- Determination of carbohydrates
- Determination of flavonoids
- Determination of triterpenoids
- Determination of glycosides
- Determination of tannins
- Determination of saponins
- Determination of proteins
- Determination of phenols
- Determination of steroids

6.2 PHARMACOLOGICAL INVESTIGATION OF OPTIMIZED EXTRACT

A: Animals

Healthy male Wistar albino rats weighing 220–280 g will be used for the assessment of the neuropathic pain-relieving activity

B: Drug and treatment schedule

Rats will be divided into eight different groups comprising of 6 rats each (n = 6). Optimized extracts will be suspended in the 1% Tween 80 solution for administration purpose and administered in graded doses of 200 and 400 mg/kg. Normal saline, gabapentin, and test extract will be administered orally to the animals for 21 consecutive days after surgery.[97, 120]

- **Group A (Normal control):** (Without any administration or surgery)
- **Group B (Reference control):** only 30 mg/kg of Gabapentin (per p.o.)
- **Group C (control):** only 10 mL/kg of normal saline (per p.o.)
- **Group D (sham control):** 10 mL/kg of normal saline (p.o.)
- **Group E (ligated control):** 10 mL/kg of normal saline (p.o.)
- **Group F (ligated reference):** 30 mg/kg of Gabapentin
- **Group G (ligated Test-I):** Low dose of Optimized extracted phytochemicals (200 mg/kg)
- **Group H (ligated Test-II):** High dose of Optimized extracted phytochemicals (400 mg/kg)

C: Experimental model for inducing neuropathic pain

Peripheral neuropathy will be induced by chronic constriction of the major sciatic nerve (CCI). In brief, in this method rats are anesthetized with ketamine hydrochloride (100 mg/kg, intraperitoneal injection [i.p]). The hairs on the lower back and thigh of the rats are shaved. The skin of the lateral surface of the right thighs is incised, and cuts are made bluntly through the biceps. The sciatic nerves are then exposed and freed from adhering tissues. Four ligatures (silk sutures size 4-0) are placed loosely around each sciatic nerve without disrupting the epineural blood supply. After performing the ligation, muscular and skin layers are sutured in layers with catgut and suturing thread respectively. Sham operations are performed similarly to the procedure described above except placing suture silk around the sciatic nerve. Following this, the rats are allowed to recover with an ad libitum supply of food and water.[121]

D: Screening methods of nociception

I.Physical parameter: Water intake, food intake, Bodyweight

II.Assessment of Behavioural parameters.[122]

- Spontaneous pain
- Allodynia: Mechanical and Thermal
- Hyperalgesia: Mechanical and Thermal

III. Assessment of Biochemical Parameters

- Estimation of lipid peroxidation levels.[123]
- Estimation of Super Oxide Dismutase (SOD).[107]
- Estimation of Glutathione (GSH) levels. [107]
- Estimation of total protein levels.[124]
- Estimation of GABA and melatonin levels.[125]
- Estimation of Serotonin level.[107]
- Estimation of Inflammatory mediators by ELIZA (TNF-α, NFkB, IL).[107]

IV. Histopathology

V. Immunofluorescence microscopy

E. Statistical Analysis (ANOVA)

CHAPTER 7: REFERENCE

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